Technical Tips Online, Vol. 3, 1998

Addition of second primer after initial denaturation step in PCR cycling: a simple way to avoid low-molecular-weight non-specific products

Marina Kniazeva, Jon Zawistowski and Min Han

MCD Biology Department, University of Colorado at Boulder, Boulder, CO, USA

Keywords: Polymerase chain reaction

▼Among the problems associated with PCR is the generation of low-molecular-weight non-specific fragments that compete efficiently with the synthesis of specific PCR products. The best way to overcome the problem is to design another set of primers that would anneal strongly to the sequence of interest. However, this approach is not always open, for instance, if only limited information is available about the template sequence. We have found that the addition of a second primer into the PCR mixture after the initial denaturation step helps to avoid the preferential amplification of non-specific PCR fragments.

We have used a pair of primers:

5′-tctctttcaccacctcttcc- 3′

5′-ttgaatgaaagttgagaagc- 3′

to amplify one of the exons of human photoreceptor matrix protein under the following conditions:

1 × PCR buffer pH 9.2
1.25 mM dNTPs
200 μM primers
0.5 units Taq-pol
1.5 mM $MgCl_2$
30 nM chromosomal DNA

30 cycles:

95°C 30 seconds
54°C 30 seconds
72°C 20 seconds

with initial denaturation and final elongation steps for 5 minutes each.

The expected size of the generated PCR product was 150 bp. However, the only fragment liberally amplified by PCR was observed on the agarose gel as a band approximately 80 bp (Fig. 1, lane 1). Direct sequence of the product has shown that the band corresponds to a non-specific fragment that contains sequences for both primers (bold), with sequence unrelated to the gene sequence in between:

5′-**tctctttcaccacctcttcc**ccgtggctnnnccgtctctttgcca...

aacttctcttcnnnatgtccag**gcttctcaactttcattcaa**-3′

A set of optimization reactions involving a combination of different $MgCl_2$ concentrations, changes of annealing temperature and varying concentrations of template and primers continually led to the same effect (not shown).

In order to overcome the problem we performed PCR under the described conditions but with the exception that just one primer was added to the PCR mix, the second primer (reverse, in this case) being added after five minutes of initial denaturation. This simple manipulation yielded greatly improved results (Fig. 1, lane 2). The non-specific band was still visible but significantly reduced, and a specific PCR product was generated. Similar improvement was obtained with at least four different pairs of primers. The rationale of this correction is to prevent imperfect match formation between primers and template at low temperature, before the cycling with the high annealing temperature begins. The same principle is the basis of the Hot Start PCR technique suggested by Perkin-Elmer [AmpliWax PCR

Corresponding author: marinak@colorado.edu

 PII: S1366-2120(08)70104-1

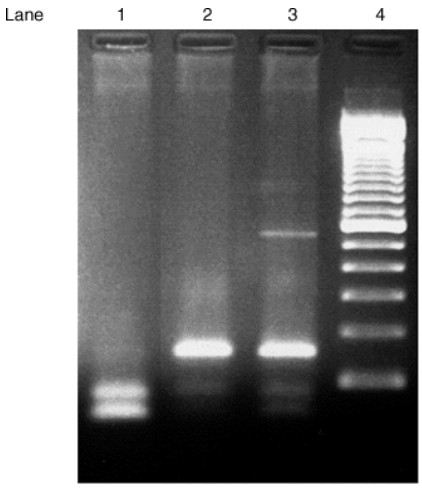

FIGURE 1. Generation of a specific PCR product as a result of addition of second primer after initial denaturation. Samples were run on a 1.6% agarose gel and a 100 bp DNA ladder (Life Technologies Gibco BRL) was run in lane 4. Low-molecular-weight non-specific product remains the major product after normal PCR mix preparation (lane 1). Upon addition of the second primer after initial denaturation, specific product is liberally amplified and a decrease in non-specific product is observed (lane 2). Addition of Taq polymerase after initial denaturation (lane 3) yields a high-molecular-weight non-specific product in addition to the specific product.

Gem 100 kit, (Perkin-Elmer)]. In Hot Start PCR, all reagents except Taq-polymerase are mixed together at room temperature and then the enzyme is added to a tube when the thermal cycler is heated to 70–80°C (Fig. 1, lane 3). However, in certain applications when commercialy available PCR mix already containing Taq-polymerase is used [PCR SuperMix (GIBCO BRL)], or the volume of Taq-polymerase to be added is less than 0.5 μl, Hot Start is either impossible or inconvenient. Another way to increase the sensitivity and specificity of PCR is known as pre-amplification heating (Ref. 1). In this method, an assembly of the reaction mixture is performed at 70°C and followed by the addition of denatured template. Compared with this procedure, our routine is more favorable because only one component (the second primer) must be added to the mixture maintained in the heated block. This reduces possible error associated with troublesome aliquoting of small amounts of reagents in fixed tubes.

We have shown that addition of the second primer to pre-made PCR mixture, after the initial denaturation step, can greatly promote synthesis of a specific PCR product. It largely reduces amplification of low-molecular-weight non-specific PCR fragments and primer–dimer amplification (not shown). We would highly recommend this very simple alteration of PCR setup as an initial remedy when problems occur.

Reference

1 D'Aquila, R.T. *et al.* (1991) *Nucleic Acids Res.* 19, 3749.

Products Used

AmpliWax PCR gems: AmpliWax PCR gems from PE Applied Biosystems

AmpliWax PCR gems: AmpliWax PCR gems from PE Applied Biosystems

PCR SuperMix: PCR SuperMix from Life Technologies (Gibco BRL)

DNA Ladders: DNA Ladders from Boehringer Mannheim